

Antibiotic susceptibility pattern among *Staphylococcus* spp. with emphasis on detection of *mecA* gene in methicillin resistant *Staphylococcus aureus* isolates

Fateh Rahimi^{1*}, Majid Bouzari¹, Zohreh Maleki², Fermisk Rahimi³

¹Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran

²Department of Pathology, Tehran University of Medical Science, Imam Khomeini Hospital, Tehran, Iran

³Department of Computer Engineering, Islamic Azad University

ABSTRACT

Background: *Staphylococcus aureus* is a major pathogen in hospital setting and in the community and causes a wide range of diseases. MRSA infection has recently become a serious problem in anti-microbial chemotherapy. The aim of the study was to detect and analyze the antibiotic diversity and isolation of methicillin resistance gene (*mecA*) of *S. aureus* isolated from Tehran hospitals as a rapid and reliable method.

Patients and methods: We studied 585 isolates of *staphylococcus* spp. recovered from patients at 3 clinical centers in Tehran from October 2005 to October 2006. Antibiotic susceptibility test of isolates was achieved with 13 antibiotics by disc diffusion. The MIC of methicillin was also performed by broth micro dilution assay. PCR was used for detection of *mecA* gene.

Results: Totally, 321 (54.7%) isolates were identified as *S. aureus*. 66, 65, 88, 88, 100, 41, 38, 41, 0, 40, 93, 20 and 64% of *S. aureus* isolates were resistant to kanamycin, cephotaxim, methicillin, oxacillin, ampicillin, erythromycin, clindamycin, sulphamethoxazole-trimethoprim, vancomycin, chloramphenicol, ciprofloxacin, gentamicin and tetracycline, respectively. All MRSA and 63% of intermediate isolates carried *mecA* gene.

Conclusion: In contrary to other studies in Iran, the prevalence of methicillin resistance is rising up in Tehran and most of MRSA isolates were resistance to 5 antibiotics at least. Vancomycin, chloramphenicol, gentamicin and clindamycin are the most effective antibiotics. All MRSA isolates had *mecA* gene with different expression. Detection of *mecA* gene is a rapid and reliable method for identification of MRSA isolates.

Keywords: *Staphylococcus aureus*, Methicillin, MRSA, *mecA*.

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INTRODUCTION

Before the advent of antibiotic therapy, invasive staphylococcal infection was often fatal. The bacterium *Staphylococcus aureus* has since demonstrated a remarkable ability to adapt to

antibiotic pressure. Methicillin-resistant strains of *S. aureus*, termed MRSA, are those strains that have acquired the ability to grow in the presence of methylpenicillins and derivatives, including methicillin, oxacillin, and nafcillin. This methicillin resistance is mediated by the acquisition and expression of an altered penicillin-binding protein, PBP2a (PBP2'), which exhibits a decreased affinity

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Reprint or Correspondence: Fateh Rahimi, Ph.D.

Department of Biology, Faculty of Science, University of Isfahan, Hezarjarib Street, Isfahan, Iran.

E-mail: fateh_rahimi@sci.ui.ac.ir

for β -lactam antibiotics (1,2). Penicillin-binding proteins (PBPs) are essential enzymes that catalyze transpeptidation crosslinking of peptidoglycan in the bacterial cell wall and are the targets of the antibiotic methicillin in sensitive strains of *S. aureus*. Inhibition of this reaction with methicillin results in the arrest of cell wall biosynthesis, triggering death of the organism through induction of the autolytic response (3). MRSA possess a 21-67 kb DNA sequence that encodes, among other things, PBP2a and genes for regulation of its expression. Methicillin-susceptible strains are inhibited by oxacillin at concentrations of 4 $\mu\text{g/mL}$ or methicillin at 8 $\mu\text{g/mL}$. In contrast, MRSA grow in the presence of 16 $\mu\text{g/mL}$ to over 2000 $\mu\text{g/mL}$ of methicillin (4).

Staphylococci cause a variety of infections, ranging from skin and soft-tissue infections to bloodstream infections and endocarditis, and the pathogenesis of these infections is well described (5-7).

Infections caused by methicillin- or oxacillin-resistant *S. aureus* (MRSA) are mainly nosocomial and are increasingly reported from many countries worldwide (8). As MRSA strains are frequently resistant to many different classes of antimicrobial drugs, second- and third-line antimicrobial resistance is a growing concern (9). Surveillance of MRSA provides relevant information on the extent of the MRSA epidemic, identifies priorities for infection control and the need for adjustments in antimicrobial drug policy, and guides intervention programs (10,11).

Methicillin, a β -lactam effective against penicillin-resistant *S. aureus* strains, became widely available in 1960. Like the development of penicillin resistance, within a year of its introduction, MRSA were reported in the United Kingdom (12,13). Sporadic reports of clinical isolates of MRSA were soon observed in the United States (14), with the first well-documented outbreak in the United States in 1968 (15). These MRSA were resistant to the entire class of β -

lactams. Today, MRSA are present in the hospitals of most countries and are often resistant to several antibiotics. Clinical infections are most common in patients in hospital intensive care units, nursing homes, and other chronic care facilities; however, MRSA are emerging as an important community acquired pathogen as well. Currently, most MRSA are susceptible to the glycopeptides, such as vancomycin and teicoplanin; however, as resistance to these agents increases, some staphylococcal infections could be untreatable (4).

Although the clinical significance of methicillin-resistance has been questioned in the past, there is now a widespread acknowledgment of the pathogenicity of MRSA. It has emerged as a significant cause of both nosocomial and community-acquired infections. Furthermore, during the past decade there has been a steady increase in the incidence of infections caused by this bacterium (16,17).

In the present study, we demonstrate our experience with antimicrobial susceptibility testing among different species of *staphylococcus* spp. and detection of *mecA* of *S. aureus* isolated from patients in 2005-2006 in selected hospitals in Tehran, capital of Iran.

PATIENTS and METHODS

Sample collection: Samples were collected during 2005 and 2006 from three different hospitals and medical laboratory in different parts of Tehran including Imam Khomeini Hospital (west of Tehran), Dr. Shariati Hospital (west of Tehran), and Negin Medical Laboratory (north of Tehran) with the associated number of isolates 252, 238 and 95, respectively.

Isolation of Staphylococci: All of the isolates were reconfirmed as *Staphylococcus* genus with different biochemical tests such as gram staining, catalase, oxidase, and growth at 10% and 15% NaCl. Presumed *Staphylococcus* was defined as the isolate that grew at 10-15%NaCl and were catalase,

gram positive and oxidase negative. The isolates were further analyzed (18,19).

Phenotyping of Enterococcal isolates: Isolates exhibiting *Staphylococcus* characteristics were identified to the species level using the following biochemical tests: acid production of xylose, cellobiose, manitol, mannose, maltose, sucrose and trehalose, alkaline phosphatase, ornithin decarboxylase, urease, Voges-Proskauer, coagulase, haemolysis, pigmentation, Pyrrolidonyl aminopeptidase (PYR) (18,19). All of different tests were compared with different negative and positive controls.

Antimicrobial susceptibility testing: Susceptibility tests were performed with disk diffusion method and interpreted according to the guidelines from the Clinical and Laboratory Standards Institute (CLSI) (20). The following antibiotics were purchased from Bio-Rad (Bio-Rad Sensi-Disc, Hercules, Calif, USA); kanamycin (30µg), cephalexin (30µg), methicillin (5µg), oxacillin (1µg), ampicillin (10µg), erythromycin (15µg), clindamycin (2µg), sulphamethoxazole-trimethoprim (1.25, 23.75µg), vancomycin (30µg), chloramphenicol (30µg), ciprofloxacin (30µg), gentamicin (10µg) and tetracycline (30µg). Minimum inhibitory concentration (MIC) of the methicillin resistant staphylococci (MRS) isolates was determined by using broth micro dilution assay (Sigma Co. St. Louis, USA). *S. aureus* ATCC 29213 and ATCC 51153 strains were used as the quality control strains (21).

Polymerase chain reaction (PCR): *mecA* gene, was detected by a modified PCR assay using the primers 5'AAAATCGATGGTAAAGGT TGGC3', 5' AGTTCTGCAGTACCGGAT TTGC3' (17).

Primer was selected from public databases and synthesized by TibMolbiol (Berlin, Germany). Primer length and G_C content were kept as uniform in reference as possible to minimize differences in annealing temperature and were checked for specificity in a BLAST search available through the National Center for

Biotechnology Information website (www.ncbi.nlm.nih.gov). For DNA extraction, one isolated colony from each plate was transferred to 200µl distilled water and boiled for 15 min at 100°C (17). The mixture was centrifuged and 10µl of supernatant were used as the DNA template in the PCR mixture (for PCR and multiplex PCR) containing; 10X PCR buffer, taq DNA polymerase (0.5U) (HT Biotechnology, Cambridge, United Kingdom), each primer (1.6µM), MgCl₂ (1.2µM) and each dNTP (0.64µM). The PCR cycles for the isolates were as follow: an initial denaturation at 94°C for 3 min, with 30 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s and elongation at 72°C for 30s and final extension at 72°C for 4 min (17). PCR products were electrophoresed on a 1.5% agarose gel in a 0.5X Tris-Borate-EDTA (TBE) buffer and stained in ethidium bromide.

RESULTS

A total of 585 isolates were identified to the species level using the standard biochemical tests. Four staphylococcal species, *S. aureus*, *S. epidermidis*, *S. saprophyticus*, and *S. haemolyticus*, were isolated. *S. aureus* was the most frequently identified *Staphylococcus* spp (54.8%) followed by *S. epidermidis* (26.7%), *S. saprophyticus* (17.8%) and *S. haemolyticus* (0.7%).

Table 1. Number and percentage of antibiotic resistance

Antibiotic	Percent	Number
Ampicillin	99	578
Oxacillin	78	456
Methicillin	78	456
Vancomycin	0	0
Erythromycin	38	225
Tetracycline	61	357
Chloramphenicol	36	210
Cefotaxime	62	362
Kanamycin	62	365
Clindamycin	35	205
Gentamicin	19	114
Ciprofloxacin	87	507
Sulphamethoxazole-trimethoprim	36	211

Antimicrobial susceptibility tests showed that the highest level of resistance was observed with ampicillin, ciprofloxacin, methicillin and oxacillin, respectively (table 1). The antibiotic resistance to three major isolates is shown in table 2. All these isolates showed resistance to more than one antibiotic.

Table 2. Antibiotic resistance in different staphylococci species (%)

Antibiotic	<i>S. aureus</i>	<i>S. epidermidis</i>	<i>S. saprophyticus</i>	<i>S. haemolyticus</i>
Ampicillin	100	100	94	75
Oxacillin	88	65	66	50
Methicillin	88	65	66	50
Vancomycin	0	0	0	0
Erythromycin	41	35	37	25
Tetracycline	64	59	57	50
Chloramphenicol	40	33	30	25
Cefotaxime	65	61	54	50
Kanamycin	66	58	59	75
Clindamycin	38	34	28	25
Gentamicin	20	21	17	0
Ciprofloxacin	93	84	73	75
Sulfamethoxazole-trimethoprim	41	40	36	25

Antibiotic resistance study showed that all 321 *S. aureus* isolates were highly resistant to, ciprofloxacin, kanamycin, cefotaxime and tetracycline (table 1). The prevalence of MRS (methicillin resistant staphylococci) was 78%, and 88% of *S. aureus* isolates were resistant to methicillin. All of the isolates were susceptible to vancomycin, while none of the MRSA isolates were resistant to all studied antibiotics. The frequency of MRSA strains isolated from different centers was similar with an average of 81% MRSA from the total staphylococcal isolations (data not shown).

A total of 134 isolates were highly resistant to methicillin with MIC \geq 256 μ g/l and 25% strains were resistant to methicillin (MIC=16-128 μ g/ml). Furthermore, 5% and 14% of MRSA strains were sensitive and intermediate (MIC=2-8 μ g/ml) to methicillin test, respectively (table 3). However, 46

S. aureus isolates were susceptible to all tested antibiotics, while 100% and 93% of MRSA isolates were resistant to ampicillin and ciprofloxacin, respectively. With the exception of vancomycin, 46% of MRSA isolates were resistant to all tested antibiotics.

Table 3. MIC (μ g/ml) of methicillin in MRSA and MSSA isolates

MIC	0.25	0.5	1	2	4	8	16	32	64	128	256	512
Number	6	6	3	3	19	14	11	6	19	34	110	51
%	2	2	1	1	7	5	4	2	7	12	39	18
MIC range	MSSA MIC=0.25-1 μ g/ml			Border Line MIC=2-8 μ g/ml			MRSA MIC=16- \geq 256 μ g/ml					

MRSA: Methicillin resistant *Staphylococcus aureus*
 MSSA: Methicillin sensitive *Staphylococcus aureus*

Using boiling method for DNA extraction, PCR for detection of *mecA* gene was performed and *mecA* gene was detected in all 284 MRSA strains. Moreover, it seems that the sharpness of *mecA* gene bands is directly associated with MIC level of methicillin, since increase of MIC level will improve sharpness of bands (figure 1).

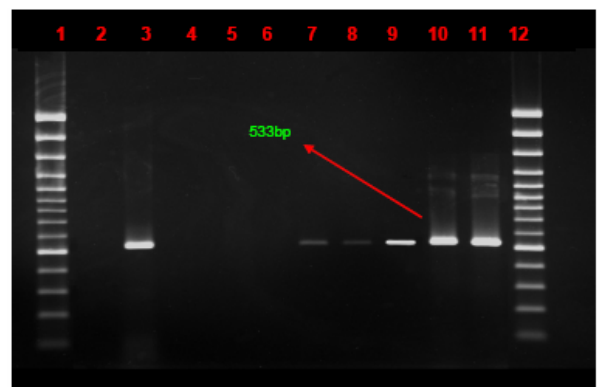


Figure 1. Correlation between sharpness of PCR band and MIC values. Line 1, 12, 100bp marker, Line 2, control negative, Line 3 control positive, Line 4-11, PCR from isolates with MIC 2, 4, 8, 16, 32, 64, 128 and \geq 256 μ g/ml, respectively

DISCUSSION

This study showed that MRSA strains have a high prevalence in Tehran. The frequency of methicillin resistance *S. aureus* strains isolated in our study was 88%. The prevalence of the MRSA obtained from different hospitals varies in different countries (6,9,12,22-24). Up to 90% MRSA, however, has been reported in Iran, where they have examined patients admitted in ICU (25). The frequency of MRSA isolated from clinical samples in Shiraz was 38% (16,17). Collectively, the data suggest that the frequency of MRSA varies in different geographical regions and the site of sample collection.

Differences between the prevalence of MRSA isolates in Iran in comparison to other countries may be in part explained by the prescription of penicillin and oxacillin for a long time in contrast to vancomycin in other countries.

MRSA infection has recently become a serious problem in anti-microbial chemotherapy. This is why during the past four decades, MRSA has spread throughout the world and has become highly endemic in many geographical areas. It has been suggested that due to the changing pattern of antibiotic resistance in *S. aureus*, it would be wise to have a periodical surveillance of these changes once every 3 to 4 years (16,17).

In this study, we could also isolate 4 different species of Staphylococci. As it was expected, *S. aureus* was the predominant species in Tehran, and *S. saprophyticus* had also high prevalence. According to antibiotic susceptibility test, it seems that *S. aureus* is the most resistant species in our study and frequency of resistance among *S. epidermidis* and *S. saprophyticus* was almost the same. We could only isolate 4 isolates of *S. haemolyticus*, thus, the obtained results in susceptibility pattern may not be reliable. Fortunately, none of these isolates were resistance to vancomycin. Similar results were reported from Shiraz (16,17).

Although chloramphenicol is a very effective antibiotic against MRSA isolates in vitro, its prescription is influenced by different side effects, a fact that could explain low resistance frequency (16,25). Sulphamethoxazole-trimethoprim is also effective against MRSA isolates. It seems that a combination regimen including sulphamethoxazole-trimethoprim may have promising outcomes.

Resistance to ciprofloxacin, tetracycline, cephalexin and kanamycin was quite higher when compared with other tested antibiotics. This may be explained by higher usage of these antibiotics. There are different evidence for transfer of tetracycline resistance plasmids between different bacterial genus and species (4).

The origins of *mecA* are obscure. A *mecA* homolog with 88% amino acid similarity to *mecA* of methicillin-resistant staphylococci has been identified in *Staphylococcus sciuri* (4,16). These and other data support the fact that *mecA* originated in a coagulase negative staphylococcus species, possibly a close evolutionary relative of *S. sciuri* (4).

The stability of *mecA* genes has been confirmed in all clinically isolated strains with MIC \geq 16 μ g/ml. These results indicate that resistant determinants are located in non-transferable DNA. If resistant genes are located in non-conjugative plasmids or chromosome, one would expect their genetic stability (26), however there are more supporting evidence that *mecA* genes are located in chromosome (17,26,27).

It is known that a variety of genes regulatory elements such as *fem* factors (factor essential for methicillin resistance), *mecI* and *mecR1* regulatory genes are involved in *mecA* expression and depression (17,26,27). It seems that isolates with MIC between 4 to 8 μ g/ml (borderline resistant) probably do not actually harbor *mecA* genes. Moreover these isolates may contain β -lactamase genes and penicillin-binding proteins which gradually hydrolyze methicillin (16,17).

PCR results showed correlation between MIC levels and sharpness of PCR bands (Fig.1). It seems that there may be conducted to copy number of *mecA* gene integrated to chromosome. This means isolates with MIC \geq 512 μ g/ml harbor a high copy number of *mecA* genes and regulatory elements (4,17). For achieving this hypothesis, all PCR tests were performed using same amount of template DNA and with similar concentration. It may also be due to the availability of sites of DNA amplification.

As described before, more than 90% of MRSA harbor *mecA* (4,28). The *mecA* gene is inducible and encodes the high-molecular-weight, 78-kD PBP_a polypeptide. It occurs in both MRSA and methicillin resistant coagulase-negative staphylococci, and is highly conserved (29-32). Analysis of the nucleotide sequence of *mecA* and its operator region revealed that sequences contained within the 5' end were similar to sequences within the β -lactamase gene, *blaZ*, of *S. aureus*.

Methicillin resistance in *S. aureus* is achieved primarily by the acquisition of an altered PBP, PBP_{2a} (also known as PBP'), which confers resistance to all β -lactams and their derivatives. *S. aureus* natively expresses four other PBPs, designated PBP1, 2, 3, and 4, that are all sensitive to β -lactam antibiotics (28). The β -lactam antibiotics serve as substrate analogs that covalently bind PBPs, inactivating them at concentrations close to the MIC. PBPs are essential proteins that are anchored to the cytoplasmic membrane and, under normal circumstances, catalyze the transpeptidation reaction that cross links bacterial cell wall peptidoglycan. Inhibition of this reaction by the binding of a β -lactam is lethal (33). Low-level resistance to β -lactam antibiotics has been observed to result from a decrease in the binding affinities of PBPs for penicillins, an increase in the production of PBPs, or both (28,34).

With respect to prior studies, it seems that the complete interpretation of *mecA* gene and relation of MIC and band sharpness needs quantitative real time PCR test. These data showed that the frequency of *mecA* gene is rising up in Iran in contrast to previous concerns studies in Iran and other countries. Achieving the real frequency of MRSA isolates in different parts of our country for treatment purposes and preventing and decreasing the nosocomial infection by these isolates as a source of acquired infection in hospitals and medical centers seems necessary. Therefore, it's necessary to detect and determine the different molecular patterns of MRSA isolated from different sources in Iran.

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